MATERIALS AND METHODS

Cell Culture

All cells were cultured at 37 °C in a 5% CO_2 atmosphere in a Thermo Forma incubator (Thermo Fisher, Waltham, MA). BEAS-2B cells (ATCC, Manassas, VA) were cultured in DMEM:F12 media (ATCC) supplemented with 10% fetal bovine serum and 50 mg/mL penicillin/streptomycin (ATCC). BEAS-2B cells were seeded at a density of 10,000 cells per well in a 96-well plate and grown to ~80% confluency for all experiments. THP-1 cells (ATCC) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (ATCC), 50 mg/mL penicillin/streptomycin, and 0.004% 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). The THP-1 monocytes were differentiated into naïve macrophages by stimulating them with 150 nM vitamin D3 (Sigma-Aldrich, St. Louis, MO) for 48 h and 10 nM phorbol 12-myristate 13-acetate (PMA) for 12 h (Sigma-Aldrich). To differentiate the naïve macrophages (M0) into classically activated macrophages (M1), they were stimulated with 10 ng/mL of LPS (Sigma-Aldrich)(Xia et al., 2013). THP-1 cells were seeded at a density of 20,000 cells per well in a 96-well plate for all experiments. Cell line authentication was performed by the Genomics Core Facility at West Virginia University, Morgantown, WV to confirm their identity.

E-liquid Flavoring Chemical Selection

An initial master list of 89 flavoring chemicals was assembled from published literature or in-house e-liquid studies (Hutzler et al., 2014; LeBouf et al., 2018; Tierney et al., 2016) and narrowed to 30 chemicals (Table 1) based on relative frequency of detection in literature of e-cigarette liquid chemical analyses, diversity of chemical structures, lack of inhalation toxicity data richness, and presence on FEMA priority lists (FEMA, 2012). Exceptions regarding diversity were made for several of the buttery flavoring chemicals (diketones), which were included either due to their known associations with severe respiratory disease or their potential to serve as

substitutes (Morgan et al., 2016; Hubbs et al., 2008). Additionally, where various versions of the same acid, aldehyde, or alcohol existed, we selected the aldehyde due to its higher reactivity and potential as a respiratory irritant (Tierney et al., 2015). Also taken into consideration was their availability for purchase through Sigma-Aldrich, whether they were available in high purity and "food grade," and whether their physical characteristics were appropriate for *in vitro* assays (i.e., not suspended in ethanol or methanol).

Preparation of E-liquid solutions

Due to low water solubility of several flavoring compounds, a vehicle solution of propylene glycol (PG) and vegetable glycerin (VG)(Glycerin Supplier, Houston, TX) was used. A 1% solution of a 50 PG/50 VG mixture was found to be sub-toxic, retained its solvent properties, and exhibited no significant difference from the PBS control for all endpoints tested. Solutions (100 mM) of flavoring chemicals were prepared at room temperature in a 100% 50/50 solution and diluted in PBS to 1% PG/VG. Concentrations of both the liquid and solid flavoring chemicals were added to the PG/VG vehicle and vortexed until the solution was homogenized at room temperature. The 100 mM flavoring chemical solutions were stored at 4 °C for the duration of the study. Cells were treated with flavoring chemicals using final concentrations of 10, 100, and 1000 µM diluted in PBS. All flavoring chemicals were classified as "Food Grade" and obtained from Sigma-Aldrich.

Cellular Viability

Viability was assessed with the alamarBlue assay (Thermo Scientific, Lenexa, KS). AlamarBlue reports viability by reacting with $FMNH_2$, $FADH_2$, NADH, NADPH, and cytochromes to measure the entire reducing potential of the cell. Cells were treated with 10, 100, and 1000 µM of flavoring chemicals and incubated for 4 and 24 hours. AlamarBlue (10 µL) was added to the medium in each well for a final volume of 100 µL and incubated for 4 hours before reading.

Fluorescence was measured at 560ex/590em with a Synergy H1 microplate reader (BioTek, Winooski, VT). Values were compared to the 1% PG/VG vehicle control.

Lactate Dehydrogenase

Membrane damage was assessed with the Homogeneous Membrane Integrity Assay (Promega, Madison, WI) according to the manufacturer's instructions. Lactate dehydrogenase (LDH) is released from damaged cells into the culture medium. The assay utilizes a coupled enzymatic reaction which converts resazurin into resorufin. The fluorescent resorufin signal is directly proportional to the amount of LDH in the media. Cells were treated with 10, 100, and 1000 µM of the flavoring chemicals and incubated for 4 and 24 hours, after which equal parts of media and reagent were mixed and incubated 10 minutes. Fluorescence was measured at 560ex/590em.

Intracellular ROS

Intracellular ROS were measured using the cell permeable dye, 2′,7′-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA is de-esterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation. Cells were incubated with the dye for 45 minutes, after which the cells were rinsed with PBS and treated with 10, 100, and 1000 µM of the flavoring chemicals. The fluorescence intensity was measured at 480ex/530em on the microplate reader to quantify the amount of ROS produced by the cells after chemical exposure for 6 hours. Separate wells of solely flavoring chemical and media were included in the plates and subtracted from their respective wells of treated cells to account for any autofluorescence.

Cytokines

Cells were grown in 96-well plates as previously indicated and treated with 1000 µM of flavoring chemical solution for 4 and 24 hours. LPS (1 µg/mL) was used as a positive control. The medium was collected and frozen at -80°C before assaying. BEAS-2B medium was undiluted for

the assay, naïve THP-1 medium was diluted 1:10, and activated THP-1 medium was diluted 1:20 in the kit diluent. The cytokine analysis was conducted according to manufacturer's instructions using the V-PLEX proinflammatory panel II (Meso Scale Diagnostics, Rockville, MD), which quantifies IL-1β, IL-6, IL-8, and TNF-α.

Methods references

The Food and Extract Manufacturers Association (FEMA) of the United States. 2012. Respiratory health and safety in the flavoring manufacturing workplace. Washington, DC.

Hubbs, A.F., Goldsmith, W., Kashon, M., Frazer, D., Mercer, R., Battelli, L., Kullman, G., Schwegler-Berry, D., Friend, S., Castranova V., 2008. Respiratory toxicologic pathology of inhaled diacetyl in sprague-dawley rats. Toxicol Pathol. 36(2): 330-344.

Hutzler, C., Paschke, M., Kruschinski, S., Henkler, F., Hahn, J., Luch, A., 2014. Chemical hazards present in liquids and vapors of electronic cigarettes. Arch. Toxicol. 88(7):1295–1308.

LeBouf, R.F., Burns, D.A., Ranpara, A., Attfield, K., Zwack, L., Stefaniak, A.B., 2018. Headspace analysis for screening of volatile organic compound profiles of electronic juice bulk material. Anal. Bioanal. Chem. 410:5951–5960.

Morgan, D.L., Jokinen, M.P., Johnson, C.L., Price, H.C., Gwinn, W.M., Bousquet, R.W., Flake, G.P., 2016. Chemical reactivity and respiratory toxicity of the α-diketone flavoring agents: 2,3-Butanedione, 2,3-Pentanedione, and 2,3-Hexanedione. Toxicol. Pathol., 44(5):763–783.

Tierney, P.A., Karpinski, C.D., Brown, J.E., Luo, W., Pankow, J.F., 2016. Flavour chemicals in electronic cigarette fluids. Tobacco Control, 25(e1), e10–e15.